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STUDIES ON THE GROWTH-PROMOTING FACTOR OF ADULT TISSUE EXTRACTS: III. PRECIPITATION WITH ALCOHOL; IV. ACTION OF LIPID SOLVENTS*

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(Received for publication on April 11, 1945)

In continuation of our studies on the growth-promoting principles contained in extracts of adult tissues (2), investigations on the growth activating power of alcohol precipitates obtained from saline extracts of adult chicken hearts were undertaken. In addition, the question was examined whether treatment with lipid solvents influences the activity of these precipitates.

A. Methods of Testing

The tests of cell growth-promoting properties of the preparations studied were performed on colonies of chicken fibroblasts. The technique employed was described in the first paper of this series (2).

B. Findings

1. *Growth-Promoting Properties of Alcohol Precipitates from Saline Extracts of Adult Chicken Hearts*

(*a*) *Preparation of the alcohol precipitates.* Freshly removed chicken hearts are cleaned of blood and fatty tissue and ground in a Latapie apparatus. The tissue pulp is suspended in four volumes of Ringer's solution, shaken for one hour and allowed to stand for 24 hours at +4° C. The suspension is squeezed through a linen cloth and the fluid is precipitated with an equal volume of 96 per cent alcohol. The material is cleared of solid particles by centrifugation at 3000 r.p.m. for five minutes. The supernatant fluid is decanted and the precipitate is spread out in open Petri dishes and dried *in*

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vacuo over calcium chloride. The material is washed with ether and finely ground in a mortar.

For the test, 1 grm. of the powder is suspended in 8 cc of Tyrode solution which is adjusted to isotonicity by addition of double distilled water. The suspension is shaken for one hour and allowed to stand in a refrigerator at +4° C. for three days, whereby a small proportion of the precipitate goes into solution. After three days the mixture is centrifuged and the supernatant fluid is heated to 60° C. for 30 minutes.[1] After another centrifugation the supernatant fluid is pipetted off. The dry weight and the total nitrogen content of the solutions previous to and after heating are indicated in Table 1.

TABLE 1

SUMMARY OF DATA ON TWO SAMPLES OF SOLUTIONS OF ALCOHOL PRECIPITATES OBTAINED FROM ADULT CHICKEN HEART EXTRACTS, BEFORE AND AFTER HEATING TO 60° C

Sample No.	Dry weight*		Total nitrogen**	
	Before heating Per cent	After heating Per cent	Before heating Per cent	After heating Percent
I	1.8	1.7	0.07	0.048
II	2.16	1.87	0.075	0.05

*Solutions were dried to constant weight of 100° C.
**Total nitrogen was estimated by direct Nesslerization method of Folin and Denis.

(*b*) *Results.* The results of these experiments are recorded in Table 2.

The mean stimulation of fibroblast cultures by solutions of alcohol precipitates of adult chicken heart extracts averages 317 per cent. The range of stimulation varies from 91 per cent to 680 per cent. The stimulation observed corresponds to that which was obtained in experiments with native saline heart extracts (3).

In the above procedure washing with ether was used to accelerate drying. This treatment is not, however, essential. The curves in Figure 1 show that alcohol precipitates which had not been washed with ether stimulate the growth of cell colonies to a high degree.

2. *Growth-Promoting Properties of Alcohol Precipitates Extracted with Lipid Solvents*

(*a*) *Method of extraction.* Alcohol precipitates prepared from

[1]This procedure is usually sufficient to make the solution bacteria-free and does not affect its growth-stimulating power (2).

TABLE 2

THE AREA OF 6-DAYS OLD FIBROBLAST COLONIES GROWING IN A MEDIUM CONTAINING SOLUTIONS OF ALCOHOL PRECIPITATES OF ADULT CHICKEN HEART EXTRACTS COMPARED TO CONTROLS IN PROTECTIVE MEDIUM

Culture No.	Size of culture in mm²	
	Control	Experiment
12746/7 *a*	8	16
b	13	58
12748/9 *a*	11	39
b	13	51
12754/5 *a*	10	58
b	13	47
12756/7 *a*	16	52
b	22	42
12773/3 *a*	19	43
b	17	36
12758/9 *a*	13	78
b	14	104
12762/3 *a*	13	79
b	12	83
12788/9 *a*	13	70
b	13	74
12776/7	22	57
12778/9	22	72
12730/1 *a*	15	32
b	17	44
12551/2 *a*	15	104
b	25	78
12593/4 *a*	7	46
b	8	52
12632/3 *a*	13	47
b	18	71
12634/5 *a*	16	113
b	18	110
12670/1 *a*	14	48
b	13	47
12672/3 *a*	10	78
b	13	52
12674/5 *a*	10	34
b	17	30
12676/7 *a*	11	39
b	13	60
Mean	14.4	60.1

saline extracts of adult chicken hearts according to the procedure described above constituted the starting material. The extraction was carried out with (*a*) ether, (*b*) petroleum-ether, and (*c*) acetone in the Soxhlet apparatus for three hours. The material obtained was dried *in vacuo* and taken up in Tyrode solution as described in the previous paragraph.

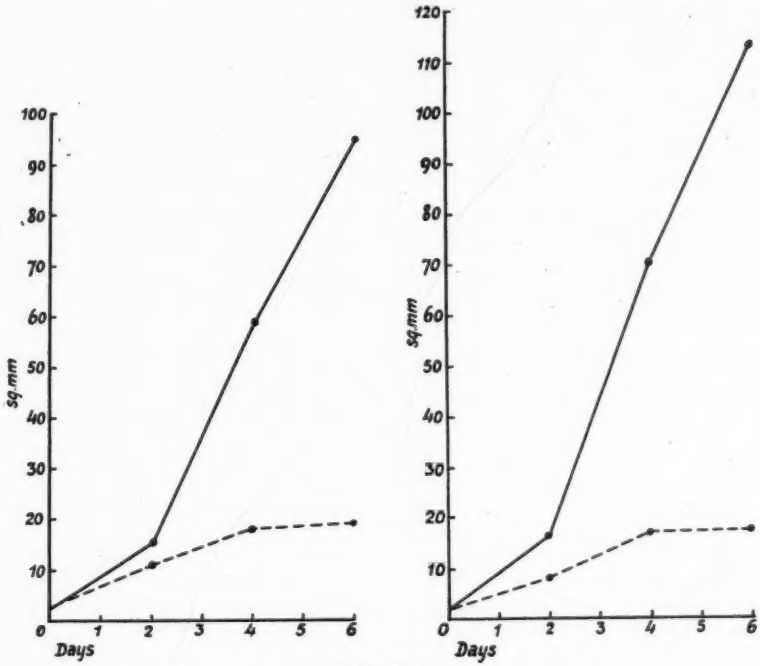

FIGURE 1

Experiments No. 12912/3 *a* and *b*. Growth of sister halves cultures in protective medium (broken curves) and in medium containing solutions of alcohol precipitates which have not been washed with ether (full curves).

TABLE 3

THE AREA OF 6-DAYS OLD FIBROBLAST COLONIES GROWING IN A MEDIUM CONTAINING SOLUTIONS OF ALCOHOL PRECIPITATES TREATED WITH ETHER, COMPARED TO CONTROLS IN PROTECTIVE MEDIUM

Culture No.	Size of culture in mm^2	
	Control	Experiment
11061/2 *a*	9	41
b	11	55
11067/8 *a*	18	53
b	16	67
11069/70*a*	14	54
b	12	54
Mean	13.3	54

TABLE 4

THE AREA OF 6-DAYS OLD FIBROBLAST COLONIES GROWING IN A MEDIUM CONTAINING SOLUTIONS OF ALCOHOL PRECIPITATES TREATED WITH PETROLEUM-ETHER, COMPARED TO CONTROLS IN PROTECTIVE MEDIUM

Culture No.	Size of culture in mm²	
	Control	Experiment
11086/7 *a*	10	68
b	15	68
11088/9 *a*	14	58
b	12	61
11090/1 *a*	19	66
b	15	98
Mean	14.1	69.5

TABLE 5

THE AREA OF 6-DAYS OLD FIBROBLAST COLONIES GROWING IN A MEDIUM CONTAINING SOLUTIONS OF ALCOHOL PRECIPITATES TREATED WITH ACETONE, COMPARED TO CONTROLS IN PROTECTIVE MEDIUM

Culture No.	Size of culture in mm²	
	Control	Experiment
11080/1 *a*	14	69
b	12	55
11082/3 *a*	8	58
b	12	68
11084/5 *a*	14	56
b	15	43
Mean	12.5	58.1

(*b*) *Results.* The results of these experiments are summarized in Tables 3, 4, and 5. They show that the alcohol precipitates of adult chicken heart extracts retain their full growth-stimulating power after treatment with ether, petroleum-ether or acetone.

C. Discussion and Conclusions

Baker and Carrel (1) precipitated the proteins of embryonic tissue extracts in a variety of ways. It was found that protein precipitates which had again been brought into solution showed decided growth-stimulating activity. Experiments were successful using carbon dioxide, ethyl alcohol, methyl alcohol, isopropyl alcohol, acetone, and acetic acid.

Fischer (4) precipitated the proteins of the embryonic extract with 96 per cent alcohol and subsequently treated them with ether. The material so obtained was found to be quite as active as the original extract.

The findings reported in the present investigations on adult tissue extracts are in conformity with the results obtained with embryonic tissue extracts by Baker and Carrel and by Fischer. They may be summarized as follows: (*a*) The growth-promoting principle in saline extracts of adult chicken heart can be precipitated with alcohol. Brought again into solution this precipitate displays a considerable growth-activating power. (*b*) The growth-promoting activity of the alcohol precipitates of adult chicken heart is retained after continuous extraction with ether, petroleum-ether, or acetone.

D. Summary

The growth-promoting principle in saline extract of adult chicken heart can be precipitated with alcohol. Brought again into solution this precipitate displays a considerable growth-activating power. The growth-promoting activity of the alcohol precipitates of adult chicken heart is retained after continuous extraction with ether, petroleum-ether, or acetone.

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GROWTH PROCESSES OF CORALLORHIZA

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The growth of a plant stem and its elongation afford an opportunity to observe phenomena concerned with meristems and their activities. The present paper contains an analysis of the growth and development of the flower-bearing axis of a plant from which it has been possible to formulate some new and fundamental concepts of development.

Growth is basically conditioned by the flow of nutritive material to embryonic or meristematic cell masses and its continued availability to derivatives during that duration. Material for the growth of organs of green seed plants may originate in all chlorophyllous tissues, in roots, or may be received from accumulated surpluses in storage in various regions. Translocation from these several sources may involve movement from base to apex, from stem to leaves, from leaves to stems, from stems to roots, roots to stems, from woody conduits to living cells, and from xylem to phloem and the reverse concurrently. Not all of these movements can be regarded as exchanges. The movement of material in solution into growing stems or flower stalks may be most definitely removed from the realm of exchanges. In these members the flow of solutions is towards transpiratory and respiratory sinks from which there is little or no return.

The building material carried in this unilateral streaming reaches the meristematic cell masses from below or behind; the final destination being the maturing derivative. The "growing zone" pushes ahead the embryonic cone, or inflorescence, in which but little enlargement takes place until the meristem at the base passes into mature tissue. Slight modification of the above program may result from preformation of flowers in resting buds, and from the amount of chlorophyll tissues in bracts, floral organs, or stems.

The completion of some studies of Corallorhiza by MacDougal and Dufrenoy (1944) placed at our disposal adequate living material and a comprehensive idea of its nutritive scheme. This plant has no roots,

their function being replaced by underground coralloid branches, and the aerial shoot is a simple scape of three or four internodes each bearing a simple sheathing scale which may have a trace of chlorophyll, so slight however, as to be of no functional consequence.

The scapes arise from vegetative cones formed in underground buds in the autumn subsequent to flowering. Anatomical examination discloses the fact that no preformation of nodes or floral organs occurs in these embryonic cones which remain in a primitive or simple condition until after awakening at the beginning of the following season. Corallorhiza, as previously mentioned, is devoid of roots, and all soil solutions are secured by the absorbing action of hyphal threads of a symbiotic fungus which ramifies in the cortical tissues and thence branches out into the soil thereby simulating root hairs. Furthermore, as has been found by MacDougal and Dufrenoy, all organic material available to the plant, hormones, vitamins, carbohydrates, proteins, phospholipids, etc. are elaborated or brought to an advanced stage of synthesis in the fungus before passing into the tissues of Corallorhiza. Carbohydrates, for example, are first recognizable in the cortex where they accumulate in quantity as starch. The hydrolysation, translocation, and repeated condensation of these substances, particularly starch, in the growing scapes where there are few living connecting elements, are unusual features which may be important factors in the course of growth of this plant.

Technique of the Experimental Work

Plants were lifted from their habitat under *Sequoia sempervirens* in the late autumn of 1943, and were established in wooden boxes of about 0.6 cu. ft. capacity containing humus and duff of the habitat. The cultures were shaded from direct sunlight, being subject to temperatures not widely different from those of the habitat.

The plant figured by MacDougal and Dufrenoy (1944, Fig. 1) brought into the culture in October, 1943, proved to be of average size for the species. The dominant bud borne at this time thrust the growing apex of the first scale above the surface early in March, 1944. Temperatures of the soil ranged from 8 to 13.5 degrees C., while the minimal (morning) temperatures of the air ranged from 8 to 17 degrees C. The final height of the scape was reached in

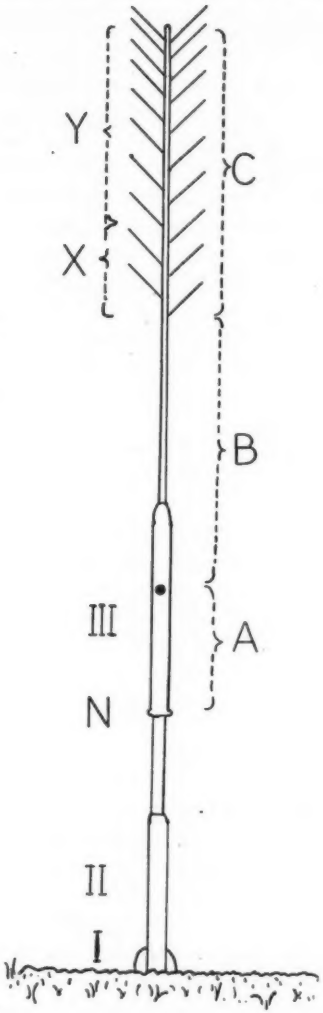

FIGURE 1

Diagram of a mature *Corallorhiza maculata* plant. I. First scale; II. second scale; III. third scale. N, node. Explanation of other reference letters is given in the text.

July when it was 70 cm. tall, bearing 41 perfect flowers, 11 of which matured seed-pods.

When the growing apex of the scale appeared above the surface in March the vertical bearing arm of an auxograph was set on the apex of the scale, and a continuous record of elongation was obtained until after the emergence of the tip of the next higher scale. The flexibility of the scape in later stages of development made further instrumental record impossible, so measurements were made directly daily.

In making a conclusive test, another resting plant of Corallorhiza was established in humus from its habitat in a suitable culture box and placed in a dark room in the winter of 1943-4. The dominant bud of this plant became active late in April and the course of growth of the combined stem, scape, and inflorescence are described in another section of this paper.

No attenuations, or changes in average dimensions of stems, or scales due to etiolation were observable. About a dozen flowers were developed in the terminal raceme of which two produced seed-pods which were mature in October. The most noticeable departure was the absence of reddish and brown coloring matter from the stem. In the tips of the petals only was some pigment to be seen. The thin sheathing scales finally became brownish but the stem and maturing seed-pods retained a creamy tint to the end. Changes in the outer layers of plants in the open, taken to be principally oxidations of tannins result in a pronounced reddish brown color (see section on etiolation).

The seeds from the two mature capsules consisting of a loose whitish membranous testa enclose yellowish ovoid embryos about 0.16 mm. in length and 0.06-0.07 mm. in thickness, which were not noticeably different from those produced by plants grown in the open. The maturation of perfect seeds of *C. maculata* constitutes the only authentic evidence that reproduction of seed-plants may take place in total darkness, and that no special substances synthesized contemporaneously by chlorophyllous tissues is necessary for the process. All of the substances necessary for the complete vegetative and reproductive processes of a seed-plant may be synthesized from compounds derived from humus by coöperating or symbiotic fungi. Growth promoting hormones and vitamins, phospholipids, proteins,

etc., may result from the metabolism in fungal hyphae and be translocated to the higher plant with which they may be associated, as described by MacDougal and Dufrenoy.

The salient features of growth of the scape of *C. maculata* in the open air have been previously described by the authors (MacDougal and Reed, 1944). There were two phases in elongation of the plant growing out-of-doors, the first started early in March and reached a maximum rate on May 13, ceased five days later, the second was somewhat irregular at the start but became fairly regular from May 19 to 29, and finally ceased June 11. Daily variations in the rate of growth appeared to be due chiefly to variations in air temperatures. The period of most rapid growth, shown by the summation curve, was in the early stage of development.

Transpiration data were obtained from two scapes separated from two other plants at base on July 12. One had an abbreviated inflorescence and the other about 30 flowers of which the lower half were open. The first weighed 11 g. and the second 13 g. The bases were stepped in small cylinders of water at once. At 5 p.m. each had taken up about 2 cc. of water in replacing dehydration in transit. The daily absorption of each plant during the five following days was approximately 0.5 cc., or about 4 per cent of the weight. No stomata were present and the loss represents vaporization from the outer walls of the epidermis.

The Pattern of Growth

The "pilot" observations on the plants growing in the open air opened the way for a series of more accurate measurements in the absence of light and under temperature and humidity having a narrower range of variation. Experience had shown that we were dealing with a unique migrating meristem, one which required a meticulous series of measurements under more uniform conditions.

1. *History of the Plant*

This Corallorhiza plant was brought from its location under the Redwoods on the north fork of the Little Sur river and established in a box filled with humus. During the course of the experiment extending from April through July the plant was examined daily by the aid of a flash-light. The daily soil temperatures ranged from 13.5

to 16 degrees C., the air temperatures ranged higher. Similar temperatures prevailed in the habitat of the plant.

2. *Experimental*

An auxograph was installed with the vertical arm bearing on the dominant bud April 10. The incipient elongation, April 16, was due to the growth of the second scale with which the enclosed axis kept pace. The first scale, sheathing the base of the bud, had attained a length of only 1 or 2 cm. and remained inactive.

During the succeeding five weeks elongation of the second scale and the enclosed axis proceeded at the following weekly amounts: 4.4, 4.8, 6.0, 5.6, and 6.0 mm. The increase for the week beginning May 22 was 8.8 mm. and in the following week it was 16.8 mm. (Table 1). On Tuesday of the eighth week at 9 a.m. the second scale opened, allowing contact with the emerging third scale. The total elongation for the eighth week was 28 mm. It was then noted that the elongation recorded was that of two meristem regions namely that of the basal internode and of the third scale which came under the bearing June 6 (Table 1). Presumably the meristem of the scale and the stem is a unified tract at first. Growth of the basal internode continued for more than a fortnight (2 mm. on June 23). The apex of the inflorescence emerged from the tip of the third scale June 19, 11 p.m.; an elongation of 4.4 mm. had ensued during the previous 12 hours. The flexibility of the inflorescence rendered further auxographic registration valueless. Observations were thereafter continued by direct measurements including the use of a specially designed sighting level. By this method it was found that on June 12 the second scale (Figure 1) had elongated 8.0 mm. since June 6.

June 22 a narrow strip was cut from the third scale (Figure 1) at a point 26 mm. above the node. An ink mark was placed on the axis at the lower end of the exposed area. The region of the axis from this ink mark to the node below was designated as A; the region of the axis from the ink mark upwards to the first pedicel as B; and the inflorescence as C (Figure 1). The growth as indicated by the increments from June 23 to 24 was mainly in the $A + B$ section, between the node and the lowest flower pedicel. The ascending meristem was passing from A into B. The inflorescence C measured 20 mm. on June 21, and while no elongation could be detected on the following

TABLE 1

RATE OF GROWTH OF CORALLORHIZA SCAPE

	Weeks ending April				May				June				July					August		
	9	16	23	30	7	14	21	28	4	11	18	25	2	9	16	23	30	6	13	20
	Increments in mm.																			
First scale	15																			
Second scale		2	4	5	6	6	6	9	17	8	1									
										Entire	B	28	67	10	0					
Third scale									20	—		79	83	27	20	14	11	12	4	
plus										Entire		C	5	27	20	14	11	12	4	
emergent												X			4	0	0	0	0	
scape												Y			16	14	11	12	4	

day, some growth was in progress, though difficult to measure (Table *A*).

TABLE *A*

	Height in mm.		
June	*A*	*B*	*C*
23	28	28	24
24	35	40	25

It is to be noted that elongation for the first six weeks was due to the joint activity of the meristem in the basal regions of the 2nd scale and of the enclosed internode, which bore at its upper end the 3rd scale with the enclosed inflorescence axis. A definitive analysis of the acceleration to 17.2 mm. in the 7th week cannot be made. Early in the 8th week the 3rd scale with its enclosures emerged. The record now comprised the results of the activity of the meristem of this final segment as well as the continuing activity of the basal internode. The node emerged from the sheathing scale in the middle of the 9th week—growth of this scale came to an end within three days, the rate of elongation dropping abruptly. The apex of the inflorescence emerged from its sheathing scale (3rd) at 11 p.m., June 19, and stood at 90 mm. above the base of the scape on a vertical meter stick; total growth of the stem was denoted by this measure until end of observations. Daily record is shown in Table 2.

The implied weekly rate is in striking contrast with the rates, which rose to 39.2 mm. in the 8th week and about 54 in the 9th week. In the 11th week it rose to 107 mm. The diminished rate of July 2 to 9 is to be coupled with the fact that the principal elongation was then in the upper part of *B*—region immediately below the inflorescence. The apex of the inflorescence rose from 179 mm. on June 27 to 250 mm. on July 4. The elongation of the sub-floral axis (Table 2) was then at an end. All subsequent growth was in the inflorescence.

On July 5 the apex of the scape stood at 256 mm., an increase of 5 mm. in total height of which about 2 mm. appeared to have been in the region of the axis immediately below the inflorescence. On the following day the inflorescence grew 4 mm., but the axis below showed no subsequent growth. On July 7 three flowers were fully open and a fourth had begun to diverge; the inflorescence showed an

TABLE 2

GROWTH OF THE TERMINAL PART OF THE SCAPE OF AN ETIOLATED *C. maculata*
Measurements in mm. (cf. Fig. 1)

		A	*B*			*C*		
			Lower	Upper	Total			Total
June	21							20
	22	26						
	23	28			28			24
	24	35			40			24
	27	53			60			25
	28	54			70			25
	29	54			75			30
	30	54	47	43	87			30
July	1	54	47	52	99			30
	2	54	47	60	107			31
	3	54	47	68	115			31
						X	Y	
	4	54	47	68				37
	5							40
	7							49
	8					31	23	54
	9					33	24	57
	10					33	26	59
	13					38	30	68
	17					38	42	80
	24					41	53	94
	31					41	64	105
August	7					41	76	117
	14					41	81	122

increase of 9 mm. The section formerly designated *C* was subdivided into *X* and *Y* and each was thereafter measured separately. *X* was the basal section extending upwards to the 5th pedicel, *Y* was the terminal section (Figure 1). The growth of each section is given in Table 2. On July 10 there were four flowers with widely divergent pedicels in section *X* and free pollen masses were evident.

A resumé of the 13th week (ending July 11) shows that a total elongation of 27 mm. occurred, all of which was in *C*., the inflorescence. The daily rate of 6 and 5 mm. in the first part of the week had fallen to 2 and 3 mm. Apparently the rate was diminishing as illustrated by the final entry in which the daily rate was less than 2 mm.

Elongation of the axis of the inflorescence *C* amounted to 21 mm. during the week ending July 17, in contrast with an increase of 27 mm. during the preceding week. Of this, 16 mm. occurred in the terminal or *Y* region of the inflorescence including the terminal pedi-

cels in which the elongation was very slight. The daily increase in *X*, the lower part of the inflorescence was 2 mm. on the 10th and fell to 1 by the 14th and zero on the 17th. The flowers on *X* were fully open with pedicels widely divergent; in *Y* the pedicels were in various stages.

As noted above, elongation continued only in the terminal or *Y* section of the inflorescence in which a number of flowers were still inactive, being appressed to the axis. Total elongation for the week ending July 24, 8 a.m., was 14 mm. in contrast with the previous week in which the total increase was 20 mm. *Y* had increased from 42 to 53 mm. in length (inclusive of the terminal flowers). Daily increases were measured as 3, 2, 1, 2, and 6 mm. for five days ending July 24. Three flowers in *X* had been retained and the ovaries appeared to be enlarging as in seed-formation. Five flowers were open in *Y* and the remaining six were beginning to diverge.

The elongation of the inflorescence in plant No. 3, in the open in which only this feature was calibrated, July 6-17, amounted to 28 mm. for the 11 day period, or 8 mm. during the week ending on the 17, during which period the developmental stages were closely parallel to those of the plant in darkness. The daily rate fell from 5 to 2 and 3 mm. Elongation was apparent only in the terminal region. This plant stood in the open subject to air temperatures of 12 to 18° C.

The method of measuring increments was changed on July 24. A mirror scale was adjusted immediately behind the tip so that its advance upward could be read easily and directly. Increases were as follows (measurements at 8 a.m. on dates given): July 25 to 31; 2 mm., 2 mm., 1 mm., 1 mm., 2 mm., 2 mm., 1 mm., total 11 mm. This was only 3 mm. less than the record for the preceding week.

Three flowers were held in *X* and the seed-pods were enlarging by an unequal growth by which the excess of growth caused a downward curvature which eventually pressed the mature pods against the scape. The pedicels have a mature length of only two or three mm. Nine flowers were open in *Y*, July 31, and were slowly diverging which may be attributed to the excess growth on the upper flank of the pedicel and pod, with the ultimate effect noted above. The terminal flower was still in a strictly vertical position, its departure from this position probably occurs at a time very near the end of elongation of the axis. Temperatures of soil and air ranged from 15.5 to 16.5° C. during this week.

Total for the 12th week of growth was 12 mm., or one more than in preceding week. This and other differences indicate some irregularity, but records are made only in mm. Some activity in the cluster of undeveloped flowers in the region was observed and one more flower had opened by August 7. The culture was watered on August 4; no immediate effect was observable. Temperatures of air and soil ranged as high as 16.5° C., about 2° higher than in the earlier stages of growth.

The above developments were slightly behind those of plants in cultures in the open, and in the habitat, in which the day temperatures were higher. Features of color remained unchanged, except that the basal scales became brownish. Some of the older pods were enlarging as if seeds were being matured.

Elongation of the scape for the week ending August 14, 1944, was 5 mm. Measurement was to tip of flower which was beginning to diverge from vertical and open. The tip of a flower next below which did not develop was taken as an index; no elongation took place, so that growth in length may be taken as coming to an end August 10 or 11. Two pods were enlarging as in seed-formation; normally only a fraction, about ¼, of the flowers are followed by maturing pods. The period of growth in length was 116-117 days. The experimental conditions departed from the normal in temperatures not varying more than two degrees, and in exclusion of sunlight. The only illumination was that coming from the daily use of a flashlight in making measurements. The final features of growth include slight extension and marked thickening of the pods. The pedicels reached their length of 2-3 mm. in an early stage.

Etiolation of Corallorhiza

The seasonal activity of shoots of green plants in the total absence of light is invariably followed by many departures in morphogenic developments, coupled with modified physiologic reactions. Size and form of organs, structure of tissues, activity of meristem with resultant rate of growth may vary widely from the normal. The majority of contributors to the subject, beginning with Ray (1686), have been prone to assume that etiolation effects arise directly from non-formation of chlorophyll or cancellation of its functions.

That the coördination is not precise or obligatory is obvious when

it is recalled that chlorophyll may be synthesized in total darkness by seedlings of pines and in fronds of ferns which at the same time display all other main etiolative effects. Furthermore, failure of reproduction by seeds is a universal effect of etiolation of green plants, but similarly spores are not formed by many fungi, including Pilobulus, Phycomyces, Mucor, and Coprinus, when mycelia are grown in darkness.

A majority of the contributions included in the vast literature deal with partial etiolations. Shoots were placed in darkness during only a part of their period of activity, or one or more branches were enclosed in dark chambers with the remainder of the shoot normally illuminated.

The disturbances resulting from the growth of green plants in darkness may be reasonably connected with the non-production of growth hormones in the usual manner, reductions of transpiration with accompanying lessened flow of sap, lessened rate of respiration; then in some cases photosynthetic products move directly to and are used in meristematic activity in neighboring regions. All material for construction in etiolated plants normally green must come from hydrolyzed and translocated surpluses. The departures from the normal in etiolation may be taken to result in the above sweeping modification of the metabolic pattern rather than to the lack of production of any specific flower, seed, or fruit-forming substances. This conclusion is confirmed by the results obtained by observations on Corallorhiza.

Corallorhiza is included in a large group of plants whose absorbing or underground organs are inhabited by symbiotic fungi which render available to the higher plant all, or nearly all, of the nutritive compounds necessary for development of the shoot and reproduction. (MacDougal and Dufrenoy, 1944). Many species do not form shoots which emerge from the surface of the loose humus which they inhabit, carrying out the entire life-cycle in an indeterminate obscurity near darkness with the development of viable seeds. No precise observations of this feature appear to have been made previously.

That many plants develop flowers, seeds and fruit well below the surface of the soil is numerously exemplified by cleistogamous species. In all such cases, however, part of the plant is exposed to the light and photosynthetic products are available.

In other cases, such as that of Epiphegus parasitic on the roots of beech trees (Leavitt, 1902), flowers and seeds are matured on underground branches; all of the leaf products of the host are, however, available to the parasite. Of especial interest in connection with the present discussion is the fact that the beech roots are characterized by a profuse and highly developed mycorrhiza and the products of the implied symbiosis would also be available to the parasite.

As demonstrated by the test described below, the material furnished by the fungus of a mycorrhiza may be adequate for the entire vegetative and reproductive cycle of the seed-plant. The record of the growth of Corallorhiza in the dark room has been given in the previous section. No attenuations, or changes in average dimensions of stems, or scales were observable in this plant. About a dozen flowers were developed in the terminal raceme of which two produced seed-pods which were mature in October.

The seeds from the two mature capsules consisting of a loose whitish membranous testa enclosed yellowish ovoid embryos about 0.16 mm. in length and 0.06-.07 mm. in thickness, which were not noticeably different from those produced by plants grown in the open.

The maturation of perfect seeds of *C. maculata* constitutes the only authentic evidence that reproduction of seed-plants may take place in total darkness, and that no special substances synthesized contemporaneously by chlorophyllous tissues is necessary for the process.

Anatomical Features

The fundamental tissues of the young scape 3 cm. high, consist principally of meristematic, polyhedral parenchymatous cells whose most conspicuous contents were starch grains, often abundant, and generally aggregated at one side of the cell (Figure 2). A minority of the cells contained clumped raphides of calcium oxalate crystals instead of starch. Intercellular spaces were very small. Nuclei were most prominent in the cells at the base of the scale rather than in those of the scape.

The application of safranin and fast green dyes colored starch grains pinkish violet, whereas the dispersed material in the raphide-containing cells was green. The raphides were often obscured by this stained material. Evidence that the contents of these young cells

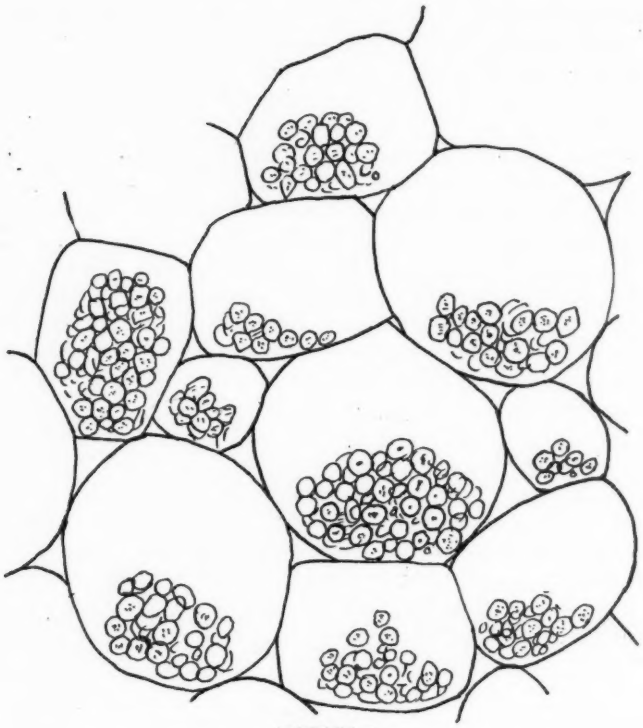

FIGURE 2

Meristematic cells from the base of a young scape. Starch grains were conspicuous.

were high in acidic substances was obtained from their behavior in aqueous neutral red solution, because their vacuoles were not stained when sections of living scapes were submerged in it. If sections of similar material were laid in phosphate buffer solutions having a pH of 6.8 to 7.7 the vacuoles absorbed the dye. On the contrary, the vacuoles of mature cells of the scapes absorbed no dye, only the walls were stained orange-yellow. Cells at the tips of young scapes had a strong reducing action on methylene blue and on thionin. Cells in the basal region were weaker in their power to reduce those dyes.

The osmotic property of these cells is physiologically important because of the character of the plant. Walter (1931) found that the osmotic strength of the sap of the basal region of *C. multiflora* was 8.6 and of the scape 5.5 atmospheres. The concentrations of sap in species of leafy green plants growing in similar conditions were greater in the leaf-bearing stems than in bulbs or roots. We found an even higher osmotic value in cells of the subterranean organ of *C. maculata*, viz. 17.8 atmospheres (by plasmolysis with KNO_3 solutions). We assume that the osmotic values were due to carbohydrates and organic acids in the vacuolar sap. Due to this high osmotic strength, it is evident to us that *C. maculata* is able to absorb water from the duff in which it lives, through its own limited epidermal surfaces and through the activity of the associated fungus.

High osmotic values in etiolated seedlings have been known since Priestley (1926) showed that the cortical cells of etiolated *Vicia Faba* plumules had higher isotonic equivalents than similar cells possessed after an exposure of only two minutes to light for three successive days. Cells of the cortical parenchyma of *V. Faba* as it differentiates from the meristem were initially packed with starch.

The sugars in two samples of *C. maculata* scapes were kindly determined by Dr. W. Z. Hassid. On a fresh weight basis the results were: (*a*) young scape—glucose 9.7 per cent, fructose 3.0 per cent, and sucrose 1.7 per cent; (*b*) old, flower-bearing scape—glucose 2.5 per cent, fructose 0.0 per cent, and sucrose 1.04 per cent. The depletion of the sugars was doubtless correlated with development of tissues and reproductive organs.

Recent advances in the knowledge of carbohydrate metabolism make it possible to link the glycogen in the hyphae of the associated fungus with the starch and sugar in the scape of Corallorhiza. It has been demonstrated that the enzyme phosphorylase acts upon glycogen in the presence of inorganic phosphate to produce glucose-l-phosphate (Cori and Cori, 1936 and 1937), and a similar enzyme system acting upon starch and inorganic phosphate produces the same compound (Hanes, 1940). Both processes are reversible. Glucose-l-phosphate is then an intermediate stage in the formation of glucose + inorganic phosphate.

Hassid (1943) concluded that the mechanisms of synthesis and transformations of starch in higher plants and of glycogen in animal

tissues and yeast are essentially similar and that the enzymic systems are also similar.

Accordingly the carbohydrates of the scape of Corallorhiza come from the glycogen in its associated fungus. Through phosphorolysis the glycogen is converted into glucose and, possibly, fructose which is translocated to the meristematic cells of the young scape. The

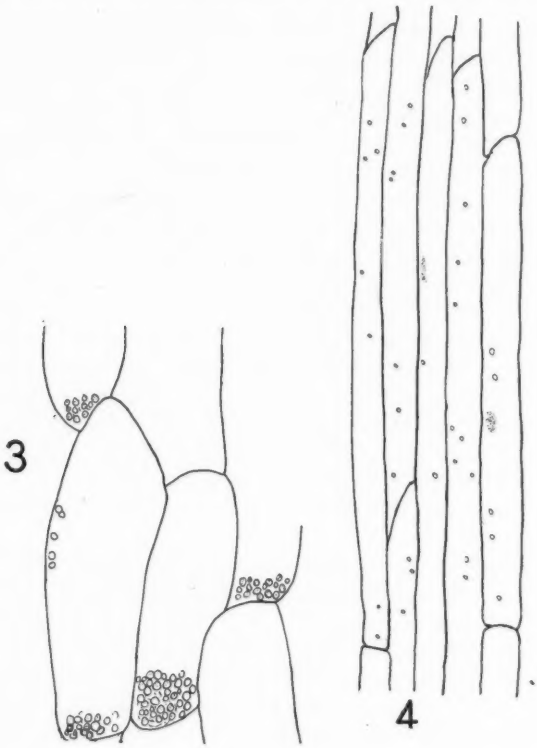

FIGURE 3
Cells from the base of an older scape. Elongation was evident.

FIGURE 4
Parenchyma cells from a region 2 cm. above the tip of the third scale. Starch grains were scarce.

phosphorylase in the said cells can reversibly transform the sugars to starch which, for a time, is a conspicuous feature.

The observations herein recorded indicate that later the starch may be converted through the intermediary glucose-l-phosphate into other hexosephosphates utilized for respiration, or other metabolic activities. Glucose, which is formed from the break-down of starch, may be resynthesized into cellulose.

Morphological differentiation was evident in the cells in the base of a scape 20 cm. high which was collected June 18, fixed in Nemec's solution and sectioned. The parenchyma cells of the basal region had grown noticeably in length (Figure 3). There were no definite intercellular spaces. Starch grains were present in the parenchyma of this stage and showed the previously mentioned tendency to aggregate. There was evidence both of the production of new starch grains and the corrosion of old grains. In the hypodermal region, long narrow cells had appeared which later became differentiated into mechanical tissue. In the young scape (20 cm. high) the cell walls were still thin, the nuclei large, and starch grains evidently corroded by enzymes which converted them into soluble carbohydrates utilizable in the rapid extension of the cells (Figure 4).

The large cells which contained amorphous basophilic material were also elongated in an axial direction and in most of them the calcium oxalate raphides were faintly visible. Their end-to-end arrangement suggested that they had also divided, but no direct proof could be obtained. At this stage starch was relatively abundant in the cells below the node, though the parenchyma cells above it contained none. The node was composed of two to four layers of short isodiametric cells, each with cytoplasm and nucleus but practically none contained starch. This nodal plate did not extend to the epidermis of the scape.

A cross-section of the basal part of the mature scape shows a structure resembling the stem of wheat (Figure 5). The epidermis was one cell thick; the hypodermal layer of parenchyma was about seven cells wide and surrounded a cylinder of mechanical tissue of varying thickness to which the scape principally owes its rigidity. There were two rings of fibro-vascular bundles; an outer in the mechanical tissue, and an inner in the fundamental parenchyma. The small individual bundles consisted largely of phloem with only about 12 tracheids.

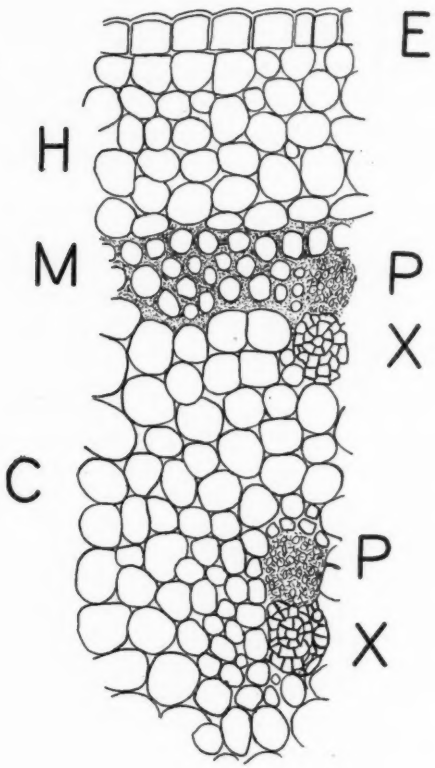

FIGURE 5
Transection of a Corallorhiza scape. E, epidermal layer; H, hypodermal parenchyma; M, mechanical tissue; C, central parenchyma; P, phloem; X, xylem.

The two-dimensional configuration of the parenchymatous cells of the scape at certain stages of development is shown in Table 3, which contains measurements made on sections of fixed and stained tissues.

The tissue at the base of the 3 cm. scape consisted of iso-diametric, meristematic cells, soon elongating in the axial direction, as shown by the measurements, eventually reaching a length seven times their

TABLE 3

Corallorhiza maculata: PROGRESSIVE CHANGES IN CELL GROWTH

Location	Average cell dimensions			
	Starch bearing		Raphide bearing	
	l	w	l	w
1. Base of scape 3 cm. tall	113	112	194	156
2. Base of scape 20 cm. tall	209	63	292	146
3. Base of mature scape	727	104	—	—
4. Node at base of upper scale	284	83	562	198
5. Node at base of young inflorescence	143	51	—	—
6. Young inflorescence enveloped in the third scale	92	38	130	52

width, and losing their starch. The cells at the base of the third scale and at the base of the inflorescence in a scape 20 cm. high contained starch and showed meristematic characters similar to those at the base of a 20 cm. scape. The cells in the young inflorescence prior to its emergence from the third scale were, however, the most characteristically meristematic of any which were measured in the upper parts of the scape. It was noted that the third scale at that stage of its development was not growing as rapidly as the regions designated *B* in Figure 1, when the terminal portion of the scape was rising on the elongating tissues below it. Elongation of *C* (Table 2) was inconsiderable until *B* had ceased to grow. The tissues of the inflorescence showed no material elongation for the first two weeks after they emerged from the scale.

In brief, the meristem of this Corallorhiza which was basal for a time, growing and pushing the apical region upwards, gradually ascended the scape. Finally, during the formative period of floral organs the meristem was in the upper part of the inflorescence.

We may remind ourselves that the upward growth of the scape was due to cell multiplication and cell elongation; of the two, the latter was the greater factor in scape growth. It is noteworthy that parenchyma cells which, in the initial stages of the scape, never increased more than twice their original length without division, grew to lengths seven times the original without division, resulting in a great increase in the ratio of boundary-surface to volume and wall area.

During the first stages of growth the cells were dividing at a rapid rate, though the mass of the scape was not greatly increased thereby.

The substrates for growth and the catalysts must have come from the subterranean perennial stem plus accretions from the fungal associates. Subsequently the growth impulse moved upwards into the second scale with its enclosed scape. The weekly increments, as measured by an auxograph, were small for six weeks, then were accelerated to a maximum of 16.8 mm., and rapidly declined to zero, in a manner observed in our former measurements (MacDougal and Reed, 1944).

The localization of the meristematic region in grasses differs from that in Corallorhiza. According to Evans and Grover (1940) the phytomers (internodes) of grass remain for a time in a highly meristematic condition. Eventually the cells of the more distal regions of each phytomer attain maturity, while most of the cells of the basal region retain their meristematic character and may continue to add new cells to the axis of the phytomer. "The axis is thus composed of intercalary meristems separated from each other by maturing or mature tissues."

The processes of translocation in the scape of Corallorhiza require some discussion. Internal factors must be considered of prime importance, since there was no significant transpirational pull on water columns. As the flow is toward metabolic centers from which there is scarcely any return, the elongation of the parenchyma cells of the growing scape enhances the osmotic movement because it lengthens the traverse between cross-walls, yet there are other important paths for the movement of liquids in this plant. The first to be mentioned is dependent on the layers of hydrophilic colloids which coat the cellulose walls and are functional in parenchyma of young tissues which contain few intercellular spaces because liquids move slowly through protoplasts. Reed and Bartholomew (1927) demonstrated the importance of external pectose layers for the flow of water through the parenchyma of young citrus fruits scantily supplied with fibrovascular bundles. Rapid fluctuations in the size of young lemon fruits are in large measure dependent on the amounts of water held by the colloidal substances in the parenchyma tissues. The importance of this process in Corallorhiza is emphasized by statements in preceding paragraphs.

Apparently Priestley (1926) was the first to call attention to the preponderating existence of carbohydrate synthesis in vacuolating dividing cells. Nothing could be plainer than the metabolic processes

involving starches and sugars in the vacuolated cells of this orchid scape, and the concomitant formation of cellulose. It seems logical to assume that termination of the growth of cells in the post-meristematic zones may result from the lack of material for the production of cellulose of lateral cell walls. So long as the cellulose lattice is filled with sap there would be no great difficulty in the transfer of solutes by this path to the active meristematic region above it. In the absence of a well developed vascular system the solute transport in the young scape must occur chiefly in these walls.

In this process of elongation the activity of the cell must necessarily be concerned with the transformation of carbohydrates into cellulose fibrils which are built into the extending walls. The situation in Corallorhiza gains in interest because having no mechanisms of its own for the production of the carbohydrates, the plant must translocate all of them from the basal region.

The determining factors in the growth of the second scale are evidently catalytic in nature, since the cells already contained starch and sugar available for cellulose production and their osmotic pressure was high enough to attract the necessary water. In fact, they served as temporary reservoirs for tissue-building materials which were utilized for the construction of the major portion of the scape. It seems logical to conclude that the catalyst of growth, which was limited in amount for the first six weeks, was suddenly increased, producing maximum growth in the ninth week, was soon exhausted and became inactive. More extended discussion of this problem will be given in a later paragraph.

An analysis of the regional growth has been given in preceding paragraphs. It will next be in order to consider some matters pertaining to the proximate causes of mobilization and construction of this part of the plant.

Corallorhiza produces no leaves or other chlorophyllous organs which could produce vitamin B_1 or similar activating substances. It is pertinent therefore to emphasize its dependence upon a fungal associate and a substratum consisting of forest duff which is densely populated with microorganisms having the ability to produce determiners of growth (Muller, 1941). The majority of green plants are able to manufacture vitamins of this class, but chlorophylless plants, like Corallorhiza, may produce none of those substances, consequently

the symbiont must manufacture or acquire from other sources the substance capable of promoting the growth of the higher plant (Melin and Lindenberg, 1939; Melin and Nyman, 1940). It is known that vitamins B_1 and B_6 occur in underground organs of Corallorhiza (MacDougal and Dufrenoy, 1944) whence they can pass to cells of the elongating scape, acting as an essential constituent of coenzymes.

The presence of auxins for transforming soluble carbohydrate into cellulose seems to be a reasonable assumption, since growth was so largely dependent upon cell-wall formation and it is known (MacDougal and Dufrenoy, 1944) that the symbiotic fungus produces indol-acetic acid in cells of the cortex where the clumps of hyphae were disintegrating.

It is probable, though not proven, that the apex of the scape also produced auxins which migrated basally to the zone in which elongation was conspicuous. The basal transport of auxins by living cells in stems has been repeatedly demonstrated in other species. Upward transport of auxins in stems has been observed only in special cases, consequently the auxins produced in the cortical cells of the subterranean stem may have a restricted zone in which they might activate growth.

Summary

The leafless scapes of *Corallorhiza maculata* arise from buds on an underground stem and produce flowers in the late summer. The aerial shoots consist of three or four internodes, each enclosed in a scale which may contain an insignificant amount of chlorophyll.

The processes of growth in *C. maculata* which have been discussed present both simple and complex problems. The growth of the shaft of tissue is dependent upon the activities of the associated fungus in the subterranean, rootless stem for carbohydrates, minerals, water, proteins, vitamins, and other compounds. The flow of nutriments is almost completely unidirectional.

The scape produces no leaves nor chlorophyll-containing cells which can assimilate carbon dioxide. Its relation to the associated fungi is parasitic rather than symbiotic. Etiolation appeared to produce no morphogenic changes in scapes or flowers. For the first time it has been demonstrated that the activities of the fungi were able

to furnish all substances necessary for normal development and seed production in total darkness.

The flow of substrates for growth pass through one and, at times, two meristematic regions. This unique physiological activity was evident during the period in which the meristem in the basal portion of the second scale and its enclosed internode were growing and in the later period when the third scale with its enclosed inflorescence axis had emerged.

The zone of active meristem migrated upward progressively, receiving the substances necessary for growth through the matured tissues below it, and carrying the terminal inflorescence upward. Elongation of the flower-bearing axis was insignificant until after the flowers opened.

Water and solutes utilized by the distal regions of the scape traversed an ascending zone of meristematic cells with but little transpirational pull.

Movement in and around cellulose walls must be regarded as two of the principal paths for translocation, though significant amounts may traverse the protoplasts. The fibrovascular elements show a weak development in the earlier stages of growth of the scape.

The tissues of the young scape consisted principally of turgid, thin-walled, parenchymatous cells, rich in starch and other carbohydrates. The cells, which were initially polyhedral, grew principally in length, retaining more or less starch until maturity. The starch and sugars were formed from the glycogen produced by the associated fungi. During the early part of its growth the scape contained significant amounts of carbohydrate in the form of sugars and starch. The formation of the mature plant was synchronous with the disappearance of those substances.

The disintegrating hyphae of the fungi liberate indol-acetic acid into the cortical cells of the subterranean stem, but other growth-promoting substances may originate in the apical region of the scape.

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A NOTE ON THE ACTION OF THE BAR SERIES IN DROSOPHILA

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In a series of interesting papers, Hersh and DeMarinis have studied the action of the Bar mutant in Drosophila by the ingenuous method of measuring eye mosaics produced by somatic crossing over. They found that the ratio of white facets to the total number conformed to the relative growth function. The analysis of the data led them to the conclusion that the Bar factors control an activator for cell division in the optic disc, the number of facets being the resultant of these divisions. In their discussion, they state that the minus effect may be brought about in three ways: namely, either by the production of a normal organ with secondary partial destruction by autolysis (claimed for the vg-series by Goldschmidt, contested by Waddington); by an agenesis of material in the mutant type; or by a failure of a normal number of cells to increase in size as in the miniature wing (Dobzhansky, Goldschmidt, Waddington). The third possibility is excluded by the normal size of the facets, the first type by Steinberg's embryological work. Hersh's own data agree with the assumption of agenesis of eye tissue. From the behavior of the mosaics, in which the time of change by crossing over can be stated, it is concluded that the agenesis means a later and still later onset of cell division in the anlage. This is illustrated in a set of theoretical curves.

Though Hersh *et al.* used different B-dosages in their experiments, they did not emphasize the dosage problem in this piece of work. But the recent investigations of Stern (and coll.) have again focused the interest upon dosage problems. Whereas both, dosage and growth problems, are interlaced in the analysis of the Bar effect, and both phases have been studied extensively in earlier work, I like to return to an earlier analysis of the situation (Goldschmidt, 1927) which now appears again to be relevant and to correct an error in this analysis which largely agrees with the results drawn from recent work.

In this discussion made from the standpoint of my conception of genic actions (p. 59ff.), I had drawn certain conclusions, using Sturtevants data, both in regard to the type of action and to its dosage. From the order of the numerical phenotypical effects in the presence of 1,2,3,4 Bar loci I concluded that an exponential function is involved which I assumed to be based upon different numbers of primary cell divisions in the eye anlage. In developing this idea, I used a sub-type of the theory of agenesis. While Hersh assumes that the time of onset of cell divisions is controlled by the number of B loci respectively (i.e., later in proportion to their number) my way of representing the facts was to assume that the reaction controlling the number of cell divisions sets in simultaneously in all cases but that their speed is proportional to the number of mutant loci, so that the threshold condition beyond which cell division stops is reached at an earlier time. In view of our ignorance of the actual cause of cell division, another way of looking at the matter would be to assume that the B alleles *decrease* the production of that growth substance which therefore is rapidly exhausted. The same process might also be assumed to occur together with a later onset of cell division in case of presence of B, namely, so low a concentration of the growth substance that the threshold of action is not reached in time as postulated by Hersh. It is clear that any of these conceptions is workable and can be expressed in a system of curves as those used by myself (1927) and by DeMarinis and Hersh (1943) respectively. Both these types of interpretation (as well as the third one just indicated) contain the same kind of general viewpoint differing only in regard to time of onset vs. time of ending of action of the division "hormone."

I used the actual data and their analysis to explain the dominance relations (though only at 25°) and to show that the reaction involved is proportional to the quantity of the "genes." In doing so, I made an arithmetical error which I never corrected but propose to do so now. My reasoning was this: If the facet forming reaction is expressed in curves (I used straight lines for a diagrammatic representation) and the number of facets of the different combinations is plotted on a line parallel to the abscissa (the threshold value) the heterozygotes should have curves intermediate between those of the homozygotes provided that only simple dosage relations are at work. Actually the group of curves were found to be arranged so that an

exponential function must be underlying their arrangement. This I assumed to be the number of cell divisions of the anlagen cells which would produce 2^n cells and subsequent facets. (The relative growth equation had not become generally known at that time.) Thus the exponent n was calculated from the actual facet number at normal temperature, e.g., 9.6 for normal eyes. Under the assumption (see above) that the lowest number of facets requires the maximum quantity of gene substance and vice versa (1B, 2B, etc.,) the relative quantity or potency of action of each mutant would be inversely proportional to the number of cell divisions. This is the point where the arithmetical error came in . Instead of calculating these proportions (which was done with 10 divisions as point 0) e.g., $n = 9.6$, dose $= \frac{10}{9.6}$, I calculated them as differences, 10—n. The value for the relative quantity of one locus is calculated from the number of facets of the homozygote and dividing this value by 2, e.g., for normal $= \frac{10}{9.6} \cdot \frac{1}{2}$. Taking these relative values for +, Bar and infrabar, all homozygous or heterozygous combinations of these, down to double infrabar and ultrabar (4 B-alleles) could be calculated and this expected result compared with n as calculated from facet numbers at 25° (Sturtevants data). Here is the corrected table which should replace the table on p. 68 of the book quoted above (Table 1).

TABLE 1

No.	Constit.	Calculated dosage	Found as 10:n.	No.	Constit.	Calculated dosage	Found as 10:n.
1	B B B B	3.28	2.18	9	Bi Bi B	2.02	1.92
2	B B B Bi	3.06	2.18	10	+ Bi B	1.94	1.76
3	B B Bi Bi	2.84	2.08	11	Bi Bi Bi	1.80	1.41
4	B Bi Bi Bi	2.62	2.08	12	+ Bi Bi	1.72	1.32
5	B B B	2.46	1.92	13	B Bi	1.42	1.61
6	Bi Bi Bi Bi	2.40	1.92	14	+ B	1.34	1.18
7	Bi B B	2.24	1.92	15	B Bi	1.12	1.05
8	+ B B	2.16	1.82				

In this table, which shows the existence of very simple dosage relations with only two small discrepancies (Nos. 9, 14) (not significant) infrabar is treated as a member of the series though it is known from the work of Luce (1926 ff.) that it has a temperature curve which is

just the opposite of that of Bar (full discussion and review in Goldschmidt, 1938). But this point does not enter here as only the optimum temperature is used. The table shows that whatever the difference between Bar and infrabar (which has not yet been clarified), the dosages of infrabar fit smoothly into the whole picture if calculated on the same basis. Thus, the table shows the simple proportionality of dosage and effect as well as the action of the alleles upon the number of primary cell divisions and thus is in harmony with the results of Hersh's analysis.

In my original analysis, I was interested in proving the actual existence of gene quantities, a view which I do not hold any longer. But nothing is changed in the discussion if the data are presented as the relation between genic dosage and quantity of effect. I think that this analysis already established the relation: mutant locus—reaction product—stimulation of cell divisions in the Bar series.

The problem which now arises is whether the details of this chain, accepted by both of us, take place according to Hersh's idea, i.e., control of time of onset by the diverse genic conditions with simultaneous end; or according to one or the other type of reasoning which I had used, e.g., simultaneous start but different end of the cell divisions. Unfortunately embryology does not furnish much information because these stages in the optic disc which precede both the temperature effective period and the first available stages of development (see Steinberg, 1941) are unknown. It is known that the onset of *facet* formation occurs later in B-flies than in +-flies while the growth curves afterwards are identical in both cases. (Steinberg's 1941; here a review is found of all former work and interpretations including one which I had preferred in 1938.) But the production of a different number of primordial cells which control—subject to environmental actions—the number of facets, called cell capital by Steinberg, occurs much earlier and is histologically unknown. It is this stage alone which is primarily controlled by the Bar mutant, and involved in the different dosage relations under discussion as all factors seem to indicate. Thus, I think, we have at present no way of knowing whether the intracellular division hormones which control ceteris paribus the facet number are produced: (*a*) so that cell division begins at a definite time, later in Bar than + (proportional to the B dosage) i.e., when a definite threshold of concentration is reached.

The alleles, in this case would control the speed of production of the division stimulant, which is inversely proportional to B-dosage; (*b*) or whether the production of the same substances always starts at the same time but is exhausted at different times in proportion to B dosage; or (*c*) whether the substance in question is inhibited by another product proportional to B dosage. The temperature experiments which show that a change is still possible after the primary differences have been established (see review in Steinberg, 1941) may be interpreted in different ways. The most recent work on the subject (Luce, 1940; Williams, 1945) shows that an increase of the time of development by means of vitamin deficiency or poisoning produces more facets (not in the case of formalin poisoning). This result may have many meanings in the light of the foregoing discussion: (*a*) Prolonged development may mean that the ending of cell divisions, which themselves are uninfluenced by the physiological condition resulting in slower development, is controlled by another developmental process which is dependent upon speed of development. (*b*) It may conversely mean that the beginning of cell division is independent of the speed of development and therefore starts at the usual time with the chance to continue longer. (*c*) It may mean that the stimulating substance is exhausted more slowly at what might be a lower metabolic level. There are other possibilities, but none of them, as far as I can see, disagree with the conclusions drawn before. Thus, I think, that we know at present (*a*) that the Bar series influences primordial cell divisions and this according to the laws of growth; (*b*) that this effect is rather perfectly proportional to dosage at normal temperature; (*c*) that growth after the establishment of the primordial cell number is independent of B dosage.

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THE DECIDUOUS DENTITION OF CHIMPANZEE

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The need, in the fields of comparative morphology and physical anthropology, for accurate indices of physical size, proportions, and rate of growth in the infrahuman primates has been frequently discussed in the literature and is too widely recognized to require further elaboration. Among such indices, rate and order of tooth eruption occupy an important place and have the added, practical, function of serving as one basis for the estimation of age in wild specimens. This paper is one of a series dealing with the early physical and behavioral development of chimpanzees. The general aims of this program, supported in part by the Samuel S. Fels Fund, have been reported (2); subsequent publications will present in detail the conditions under which these animals were raised, a variety of measures of development, and the interrelationships among the several indices. Discussion of the wider implications of the present data on dentition will be reserved until after this further body of relevant information has been made available.

Sixteen chimpanzees, nine males and seven females, separated from their mothers within two weeks after birth and raised under standardized nursery conditions, were examined for eruption of the deciduous teeth at one- to seven-day intervals. The recorded ages at which each of the 320 teeth appeared may therefore be considered accurate to within a few days. "Eruption" was arbitrarily defined as the projection of any part of the tooth one millimeter through the gum. In 34 (out of 128) cases eruption of the central and lateral incisors (mostly in the lower jaw) was preceded by extreme puffiness and tenderness of the gums which interfered with bottle feeding; to relieve this condition, the gums were slit, thus hastening the appearance of these teeth by a few days to perhaps as much as a week. In addition to the data on these 16 infants whose growth occurred under known

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and uniform conditions, observations on several other individuals with divergent environmental histories will be presented.

Results

The formula of the complete deciduous dentition in chimpanzee is the same as in man: central and lateral incisors, canine, first and second molars on each side of the mid-line in upper and lower jaws. The mean and median ages and the age-ranges for the eruption of each of the 20 teeth are shown in Table 1. The rather close corre-

TABLE 1

Age in Days at Time of Eruption of the Deciduous Teeth in 16 Chimpanzees

	9 Males				7 Females			
	Mean	Mdn.	Range	Order*	Mean	Med.	Range	Order*
Central Incisors								
Upper R	111	115	79-161	4	73	74	44-108	3
Upper L	110	115	77-161	3	79	75	40-108	4
Lower R	95	91	55-126	1	68	70	53-79	1
Lower L	96	97	60-122	2	72	76	53-83	2
Combined	103	103	55-161	I	73	74.5	40-108	I
Lateral Incisors								
Upper R	122	109	83-177	5.5	91	87	74-111	7
Upper L	122	113	86-177	5.5	97	95	83-112	8
Lower R	124	124	68-172	8	88	87	72-105	6
Lower L	123	121	70-188	7	87	81	65-112	5
Combined	123	120	68-188	II	91	91.5	65-112	II
Canines								
Upper R	356	354	266-411	18	331	324	226-445	18
Upper L	354	354	268-410	17	323	342	232-415	17
Lower R	369	376	293-432	19	352	349	272-492	20
Lower L	384	382	293-459	20	350	349	265-483	19
Combined	366	375	266-459	V	339	345.5	226-492	V
First Molars								
Upper R	127	122	78-177	10	107	109	74-147	9
Upper L	126	120	77-180	9	114	112	84-152	10
Lower R	150	149	79-210	12	136	132	106-170	12
Lower L	149	148	79-210	11	134	131	108-176	11
Combined	138	132	77-210	III	123	121.5	74-176	III
Second Molars								
Upper R	310	308	226-446	16	293	282	243-379	16
Upper L	307	300	240-425	15	288	288	243-350	15
Lower R	244	229	183-338	13	225	233	154-285	13.5
Lower L	250	237	198-338	14	225	222	162-291	13.5
Combined	278	274.5	183-446	IV	258	263	154-379	IV

*Sequence of eruption, based on mean ages. Ordinals for the five types of teeth are shown in Roman numerals.

spondence between means and medians indicates fairly normal distributions, even with these small samples, although the fact that in the majority of cases the mean is slightly greater than the median suggests a tendency towards skewness to the right or higher ages.

1. *Variability*

Absolute variability, as indicated by the ranges, is smallest for the incisors, slightly higher for the first molars, and greatest for the canines and second molars. Relative variability (roughly estimated by dividing the range by average age at eruption) is least for the canines, almost twice as great for the other four teeth. Both absolute and relative variabilities are higher for the males than for the females.

The figures of Table 1 show that eruption of the deciduous teeth may serve as only a crude guide for the estimation of age. In the case of males, the first tooth (a central incisor in 15 of the 16 cases) appears as early as the 55th day and as late as the 122nd day; in one case all 20 teeth have erupted by the 293rd day whereas in another individual dentition is not completed until the 459th day of life.

2. *Correlations with Length of Gestation*

Since the *Anlagen* of the deciduous teeth are formed early in fetal life, it is reasonable to expect a negative correlation between length of gestation and age at eruption of the teeth. Rank order correlation coefficients between gestation ages (3) and the average of eruption-age for the 20 teeth of each animal are —0.449 for the group of 16 animals, —0.512 for the nine males, and —0.241 for the seven females.

If "dental ages" are computed by adding to each individual's average tooth-eruption age the length of his gestation, it is found that the ranks of the 16 individuals in speed of dentition remain almost unchanged: the rank order correlation coefficient between "dental age" and average eruption age is +0.968. When males and females are considered separately, this coefficient becomes +1.0 and +0.857, respectively.

3. *Sex Differences*

It may be seen from Table 1 that tooth eruption occurs slightly but consistently earlier in the females than in the males. The average

value for the 180 male teeth is 201 days; for the 140 female teeth it is 177 days. In view of the correlation exhibited in the previous section, it is clear that any differences in gestation length must be taken into account in such group comparisons. The average gestation length of our nine male infants was 225 days, or 3.6 days shorter than the average of the seven females, 228.6 days. As much as 3.6 days, therefore, may be attributed to the factor of gestation length, giving a net acceleration of females over males of 20.4 days. Even this correction may be insufficient since it is conceivable that the last few weeks *in utero* may be especially critical for dental development. At any rate, the small number of cases and high variability make it impossible to ascribe statistical significance to the sex difference indicated by our figures.

4. *Sequences of Eruption*

Based on either means or medians, the order of eruption for the group as a whole is as follows; figures in parentheses are average ages in days:

I.	Central incisors	(90);	Lower	(84);	Upper	(95)
II.	Lateral incisors	(109);	Lower	(108);	Upper	(110)
III.	First molars	(131);	Upper	(120);	Lower	(143)
IV.	Second molars	(269);	Lower	(237);	Upper	(300)
V.	Canines	(354);	Upper	(343);	Lower	(365)

Deviations from the upper-lower sequence indicated above were frequent. Deviations from the sequence of the five types of teeth as exhibited by group averages were less frequent: there were five animals in whom one or two canines erupted before all four second molars had appeared, nine cases in which one to four first molars came through before all of the incisors had erupted, and nine cases of one or two lateral incisors appearing before all central incisors had pierced the gums. There were only three animals with two-rank displacements: first molars erupting before all central incisors had appeared. Three animals had no such deviations; one had all four types of displacement. There is no significant correlation between number of displacements from the group trend and average acceleration or retardation of tooth eruption, but there is a suggestion of a relation to parentage: the offspring of certain mothers, and especially of certain fathers, seem to show greater conformity to the group pattern than do the infants of other parents.

5. *Individual Consistency in Speed of Dentition*

An individual relatively accelerated in respect to the eruption of one or several teeth usually is accelerated also in respect to the rest of his deciduous dentition. For each tooth, each individual was given a rank from 1 to 16 in accordance with whether his age at eruption of that tooth was the lowest of the group, second lowest, and so on. For each individual the average of his 20 ranks was computed and the deviations from this average were calculated. These 16 average deviations range from 0.53 to 5.09, and average 2.35. The *AD* produced by chance alone would be 5.31. The statistical results, therefore, indicate a fairly high consistency for each individual in speed of eruption of the 20 teeth. It should be pointed out, however, that there are occasional exceptions of considerable magnitude. As an extreme example, animal "Falla" may be cited; she was second fastest of the group in getting the upper right lateral incisor (79 days) but was the slowest (rank 16) in erupting the four canines (upper R 445, L 415; lower R 492, L 483 days).

Especially for purposes of comparison with other growth data to be presented for these same animals, Table 2 has been prepared. For

TABLE 2

SHOWING AVERAGE AGE IN DAYS AT ERUPTION OF THE DECIDUOUS TEETH AND RANKING OF EACH INDIVIDUAL IN ITS SEX GROUP AND IN TOTAL GROUP OF 16 CHIMPANZEES

		Males					Females		
		Avge. eruption age	Rank among males	Rank among all 16			Avge. eruption age	Rank among females	Rank among all 16
Spratt	(10)*	147	1	2	Fanny	(6)*	145	1	1
Scarf	(2)	185	2	7	Jenny	(5)	151	2	3
Alf	(0)	185	3	8	Banka	(6)	168	3	4
Jed	(11)	195	4	9	Karla	(0)	177	4	5
Ken	(0)	196	5	10	Jojo	(2)	182	5	6
Jent	(6)	201	6	11	Flora	(2)	201	6	12
Art	(8)	213	7	14	Falla	(2)	212	7	13
Bard	(8)	240	8	15					
Web	(6)	251	9	16					

*Number of deviations from group order of eruption. See text.

each individual the ages at which each of the 20 teeth erupted were averaged and these figures are entered as the "average eruption age." The numbers in parentheses indicate variability from the group trend as to order of eruption of the five types of teeth (see preceding

section). Thus the number 11 for Jed is derived from the following order of eruption, involving one two-rank and 10 one-rank displacements: CI, CI, LI, LI; M1, M1, CI, M1; CI, LI, LI, M1; M2, M2, M2, C; C, M2, C, C. (Since the rank-displacement of one tooth necessarily involves the displacement of another one, it might be more accurate or logical to consider the number of displacements as being equal to one-half of the figures shown.)

6. *Effect of Environmental Conditions*

The 16 animals from whom the data of Table 1 are obtained were raised under a uniform nursery diet and routine. Prior to the inception of the Infant Studies Program, three other infants born at the Laboratories were raised from birth by human caretakers under varying conditions of diet, routine care, and experience: Alpha, Peter, and Fin. Comparable data on dentition are available for these individuals.[2] We include here also the observations of Vanderplank (7), as given by Schultz (6), on the chimpanzee Adam. Alpha, whose growth in terms of weight and a variety of anthropometric measures was relatively accelerated, was also early in eruption of the deciduous teeth. With the exception of the upper second molars (which erupted at the ages of 205 and 203 days), however, her figures are within the ranges for females shown in Table 1. Peter was relatively slow; his upper canines erupted at 428 and 430 days, his lower canines at 470 days, his upper left first molars at 181 days, and his lower ones at 220 days; his other ages are within the male ranges presented above. The data for Fin and for Adam approximate the male averages given in Table 1. The order of eruption of the five types of teeth in all four cases is the same as that shown above for the 16 nursery infants.

Some data on deciduous dentition are available also for 25 infants born at the Laboratories between 1931 and 1943 which were raised by their chimpanzee mothers for the first 6 to 14 months of life. In terms of weight increase during the first year of life these animals were markedly retarded as compared to the nursery-raised youngsters. Dental observations were made by various members of the staff as opportunity offered. Some of the mothers were fairly coöperative in allowing examination of their infants, but many others made the

[2]Observations on Alpha were made by Jacobsen, Jacobsen, and Yoshioka (1); on Peter by Drs. Haslerud, Elder, McCulloch, and Crawford; and on Fin by Dr. Glen Finch.

procedure extremely difficult if not impossible. In numerous instances our only information is that certain teeth were absent or fully erupted by a given date. A survey of these figures, insofar as they are meaningful, shows that with few exceptions they fall within the ranges of Table 1 but that they tend to be somewhat higher than the means shown here, especially in respect to the incisors and first molars and more markedly for the females than for the males. The lower limits of the ranges are exceeded by two males: Dick, whose second molars erupted before 204 days (upper) and 180 days (lower), and Bob, whose lower left second molar was in before the age of 198 days. Figures exceeding the upper limits of Table 1 ranges are as follows:

Central incisors, upper: 151, 151 (Beta), 132, 132 (Gamma), between 175 and 218 (Tim);
lower: 122, 161 (Beta)
Lateral incisors, upper: over 121 (Brinka), 186, 186 (Beta), over 132 (Gamma);
lower: 165, 165 (Beta), over 132 (Gamma)
Canines, upper: 514, 508 (Beta);
lower: 585, 567 (Beta), 590, over 590 (Tom)
First molars, upper: 192, 164 (Beta), 168 (Gamma);
lower: 180, 205 ((Beta), 185 (Gamma), over 183 (Delta)
Second molars: None

The sex of these individuals is determinable from the convention that female names end in vowels, male names in consonants.

Our tentative conclusion from these results is that, within the limits of variation here represented, the factors of diet and care do not greatly influence the speed with which deciduous dentition proceeds. The direction of the small differences which do appear in the data suggest that nursery-raised infants may be slightly accelerated in this respect as compared to infants raised by their chimpanzee mothers. The former group almost certainly consumed more food than the latter, which fact is reflected in the differential rates of weight-growth mentioned above.

7. *Comparisons with Human Deciduous Dentition*

The most recent as well as most accurate data on human deciduous dentition are those of Robinow, Richards, and Anderson (4) and will be used here for comparison with our chimpanzee records. In Table 3 the human data (4, p. 128) have been converted into days, and are presented together with the comparable figures for our chimpanzee subjects. The criterion for eruption is very similar in the two studies.

TABLE 3
COMPARISON OF HUMAN AND CHIMPANZEE DECIDUOUS DENTITION

	Males			Females		
	Human*	Chimpanzee*	Difference	Human*	Chimpanzee*	Difference
Central Incisors						
Upper	273 (2)	111 (2)	162	288 (2)	76 (2)	212
Lower	219 (1)	95 (1)	124	234 (1)	70 (1)	164
Lateral Incisors						
Upper	312 (3)	122 (3)	190	357 (3)	94 (4)	263
Lower	390 (4)	124 (4)	266	414 (4)	88 (3)	326
Canines						
Upper	567 (7)	355 (9)	212	603 (7)	327 (9)	276
Lower	579 (8)	376 (10)	203	606 (8)	351 (10)	255
First Molars						
Upper	480 (5)	126 (5)	354	471 (6)	111 (5)	360
Lower	486 (6)	150 (6)	336	468 (5)	135 (6)	333
Second Molars						
Upper	828 (10)	308 (8)	520	852 (10)	290 (8)	562
Lower	777 (9)	247 (7)	530	813 (9)	225 (7)	588

*Age in days at time of tooth eruption. Figures in parentheses indicate order in which the teeth appear. Human data from Robinow, Richards, and Anderson (4).

Human deciduous dentition begins later (219 *vs.* 70 days) and ends later (852 *vs.* 376 days) than that of the chimpanzee. The former covers a span of over 600 days, the latter a span of about 280 days. When relative variability is calculated for the chimpanzees by assuming that σ is one-sixth of the range (Table 1), estimated cofficients of variations are obtained (ranging from 7 to 16) which are roughly similar to those yielded by the human data. Sex differences are in opposite directions in the two cases, the human males being earlier than the females, whereas among our chimpanzee subjects the females generally had their teeth at an earlier age than the males. In respect to order of eruption, there is one clear-cut species difference: in man the canines erupt before the second molars, whereas in chimpanzee the canines are usually the last to appear. There are some differences, also, in regard to upper-lower sequence, particularly of the lateral incisors and second molars.

8. *Comparisons with Rhesus Monkeys*

According to Schultz (5) the average age for eruption of the deciduous teeth in rhesus monkeys (Macaca mulatta) ranges from

20 days for the central incisors to 176 days for the second molars.[3] These ages bear the same relationship to the absolute eruption-ages for chimpanzee as the latter bear to the figures for man: rhesus deciduous dentition starts earlier, is completed earlier, and covers a smaller range (156 days), than that of chimpanzee. In respect to order of eruption, it is to be noted that in the rhesus eruption of the canines is relatively advanced, approximately coinciding with that of the first molars, and being well ahead of the second molars.

The above species comparisons are based on absolute ages at the time of eruption. Additional significance would be gained if these comparisons could be expressed also in relative terms: as the ratio of average eruption-age to life-span, for instance. The only fairly accurate index of this kind available for all three species concerned is age at first menstruation; approximately 12 years in man, 8½ years in chimpanzee (8), and 3 years in the rhesus macaque (5). Dividing age at menarche by average deciduous eruption-age, the following ratios are obtained: man 0.11, rhesus 0.08, chimpanzee 0.06. On this basis, therefore, relative speed of dentition is greatest in chimpanzee, slowest in man.

Summary

The age at eruption of each of the deciduous teeth was determined for 16 chimpanzees raised under uniform conditions. The average ages in days are as follows: central incisors, 90; lateral incisors, 109; first molars, 131; second molars, 269, canines, 354.

Inter-individual variability is fairly high and there are numerous exceptions to the group trend in order of eruption. There is, however, a considerable degree of individual consistency, each animal tending to maintain a fairly constant rank within the group in speed of eruption of each of the 20 teeth. The data suggest that females are somewhat faster than males in eruption of the deciduous teeth. There is a negative correlation between length of gestation and average age at which the teeth first appear through the gums. Certain nutritional and other environmental factors do not seem to have any marked influence on speed or sequence of eruption.

[3]Since for Schultz the age at eruption refers "to the time when the entire top of the crown has just pierced the gum," these figures would be even lower if the criterion used in the present study had been applied.

In comparison with man, the deciduous dentition of chimpanzees starts earlier, is completed sooner, and covers a narrower span of time. The order of eruption in the two species is approximately the same with the notable exception that the sequence of second molars and canines is reversed. The decreasing order of relative speed of dentition appears to be: chimpanzee, rhesus monkey, man.

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SOME EFFECTS OF HEXENOLACTONE ON TISSUE CULTURES*

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A. INTRODUCTION

The synthesis by Medawar, Robinson, and Robinson (1943) and by Kuhn and Jerchel (1943) of an unsaturated lactone, δ-hexenolactone, which inhibits the growth of mesoderm in tissue cultures—a property of the inhibitory factor extracted from malt, yeast (Medawar, 1937) and *Sorbus aucuparia* (Kuhn and Jerchel, 1943)—suggested an easier method of culturing in vitro pure epithelial tissues without the tedium of the subculturing technique.

Medawar (1937) found that at the proper concentration of the naturally occurring growth inhibitor, isolated from commercial malt extract, fibroblast growth was inhibited and epithelial growth was unaffected in cultures of chick intestine, lung, metanephros, rat lung, and metanephros and carcinoma 206 which are composed of epithelial cells and fibroblasts. Kuhn, Jerchel, Moewus, Möller, and Lettre (1943) reported that the synthetic compound inhibited heart fibroblasts and Staphylococcus aureus although it had no effect on Ehrlich rat carcinoma and *Streptobacterium plantarum*.

In the work here reported various concentrations of hexenolactone[1] were tested on cultures of chick stomach and intestine, and on human mammary gland carcinoma to determine the amount required to obtain pure epithelial outgrowths, and on chick leg and heart muscle to determine the inhibitory effect on other types of mesoderm. Although a wide range of concentrations failed to elicit a significant differential inhibition on intestinal cultures, hexenolactone was not without an effect on heart and leg muscle fibroblasts. It seemed worthwhile to publish the data from these experiments since the conditions of the experiments differed from those already reported.

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[1]Synthetic hexenolactone was obtained through the courtesy of the McNeil Laboratories, Inc., Philadelphia, Pa.

B. Method

A series of 20 experiments, 5 with hanging drop and 15 with roller tube cultures, were carried out using various concentrations of a synthetic unsaturated δ-hexenolactone on a total of 1369 explants. The tested material included 18-day chick metanephros, stomach and intestine from 9, 10, 11, 12, and 14-day chick embryos, heart from 10, 11, 12, and 14-day chick embryos, leg muscle from 9, 10, and 12, and 14-day chick embryos, and a human mammary gland carcinoma.

Hexenolactone was used in the following concentrations per cc of culture medium in hanging drop and roller tube cultures: 0.5 mgm, 0.1 mgm, 0.05 mgm, 0.035 mgm, 0.025 mgm, 0.02 mgm, 0.015 mgm, and 0.011 mgm.

Each experimental hanging drop preparation consisted of three explants in an 0.02 ml clot of chicken plasma, fetal cord serum, and hexenolactone diluted in Gey's solution (Gey and Gey, 1936). Control cultures were grown in plasma, fetal cord serum, and Gey's solution without hexenolactone. All clots were about 1 cm in diameter to insure uniformity in thickness.

Roller tube preparations were cultured in a plasma clot and a supernatant fluid medium of 1 cc of human fetal cord serum and 1 cc of dilutions of hexenolactone in Gey's solution. Control cultures varied only in having 1 cc of Gey's saline without hexenolactone. In one series of experiments to test the effect of serum on the activity of the hexenolactone, Gey's saline was substituted for fetal cord serum.

The reversibility of the effect was determined by replacing experimental media with control media after three days growth under experimental conditions. This was done in six series of experiments.

Observations were made from 24 to 96 hours after the cultures had been set up. Comparative data were collected on 48-hour cultures unless otherwise noted. It was found that within 48 hours, the outgrowth of almost all control cultures extended about 3 mm from the edge of the explant. All muscle and heart outgrowths were graded according to whether they were greater or less than this width. Outgrowths of stomach, intestine, metanephros, and tumor consisted mostly of fibroblasts. Therefore, only when the epithelial outgrowths surpassed the fibroblasts was a differential inhibitory effect recorded.

C. Results

Explants grown in concentrations of hexenolactone showed one or more of the following effects: (*a*) total inhibition, (*b*) partial inhibition (heart and leg muscle cultures), (*c*) differential inhibition (mixed cultures of epithelial cells and fibroblasts), and (*d*) normal growth for 24 hours followed by a degeneration of the cells of the outgrowth.

1. *Effect on Heart and Leg Muscle Fibroblasts*

Table 1 indicates the results of various concentrations of hexeno-

TABLE 1
Effect of Hexenolactone on Heart and Leg Muscle

Concentration hexeno-lactone mgm/cc	Number of explants	Percentage explants totally inhibited	Percent-age explants degenerated	Percentage explants partially inhibited	Percent-age explants unaffected
0. 05	58	47		53	
0.035	58		28	55	17
0.025	160	20	22	16	42
0.015	31		23	42	35
0.011	33			63	37
Controls	170	1		5	94

lactone on 9-14 day chick embryo heart and leg muscle. No concentration inhibited all the growth from the explants. The concentration of 0.05 mgm/cc was the most effective one tested on these tissues in that 53 per cent of the explants in this group were partially inhibited, the remainder (47 per cent) failed to grow at all. None was without an effect. Figure 1 shows a luxuriantly growing control heart culture and Figure 2 an experimental culture from the same series grown two days in 0.05 mgm/cc hexenolactone.

At 0.035 mgm/cc concentration, no explants were completely inhibited, 55 per cent were partially inhibited, and 17 per cent were unaffected. In 28 per cent, the heart and leg muscle cultures during the first 24 hours equalled controls in the quantity and quality of outgrowth which, during the next day, underwent degenerative changes. Cells reacting in this manner accumulated an inordinate amount of fat in their cytoplasm. The cell membranes became indefinite and many cells began to decrease in volume and to disintegrate.

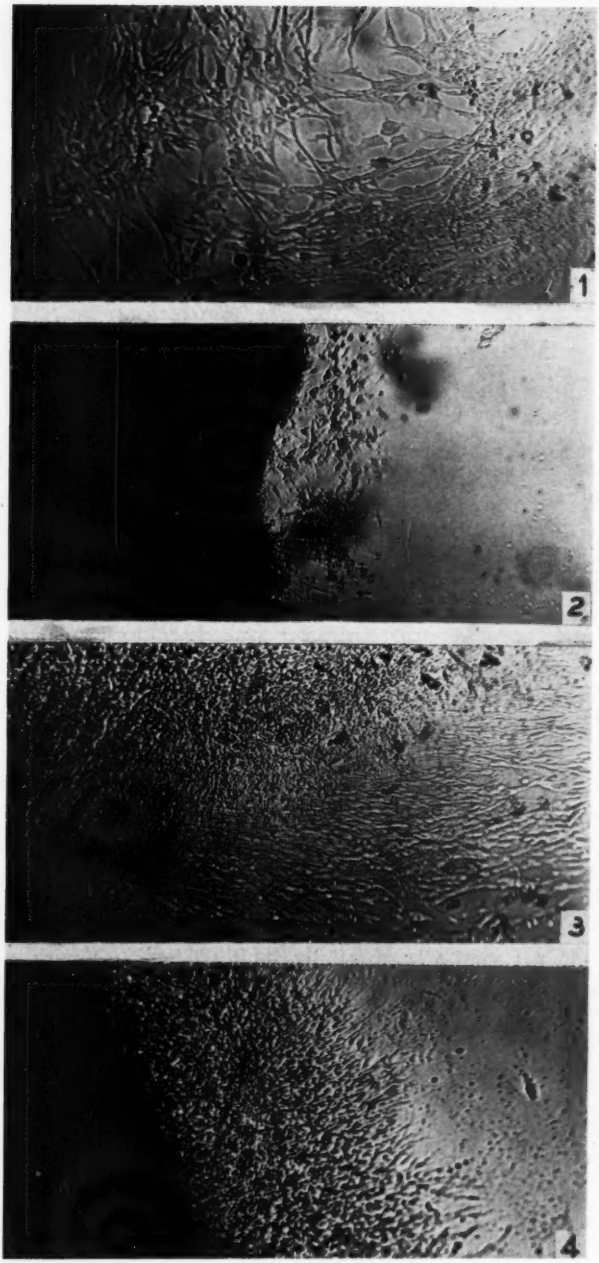

PLATE 1

Fig. 1. Control: 2-day culture of embryonic chick heart. Outgrowth is wide. x 100.
Fig. 2. 0.05 mgm/cc hexenolactone: 2-day culture of embryonic chick heart. Outgrowth is inhibited. x 100.
Fig. 3. Control: 2-day culture of embryonic chick leg muscle. Extensive zone of growth is wider than the diameter of the camera lens. x 100.
Fig. 4. 0.025 mgm/cc hexenolactone: 2-day culture of embryonic chick leg muscle. Outgrowth is much less than in the control (Fig. 3). x 100.

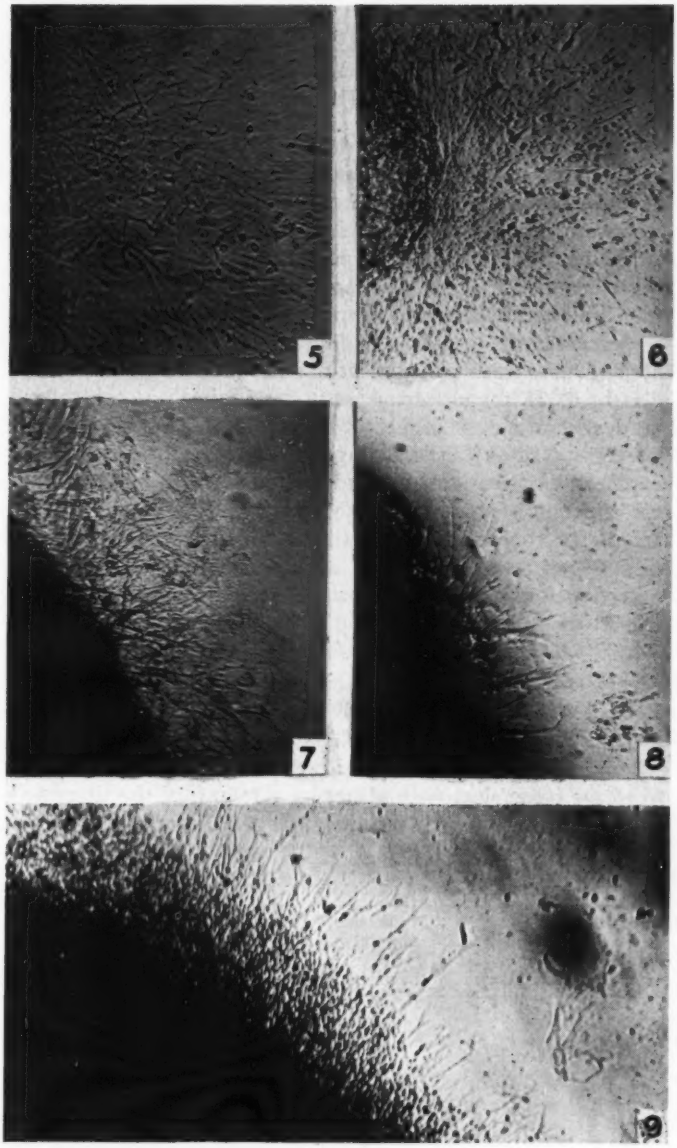

PLATE 2

Fig. 5. Control: 2-day culture of embryonic chick leg muscle. Cells are normal in appearance. x 100.

Fig. 6. 0.015 mgm/cc hexenolactone: 2-day culture of embryonic chick leg muscle. During first 24 hours cells resembled controls (Fig. 5). They subsequently became degenerate. Cells are very fatty; cell membranes are indefinite. x 100.

Fig. 7. Control: 3-day culture of a human mammary gland carcinoma. Fibroblasts grew well and surrounded the entire explant. x 100.

Fig. 8. 0.035 mgm/cc hexenolactone: 3-day culture of a mammary gland carcinoma. This small tuft of fibroblasts appeared only at this point on the explant. x 100.

Fig. 9. 0.05 mgm/cc hexenolactone: embryonic chick heart fibroblasts. Same culture as Fig. 2, but 24 hours after experimental media had been replaced with control media. New cells have grown out indicating that the inhibitory effect is reversible to some extent. x 100.

Only half of the cultures treated with 0.025 mgm/cc hexenolactone reacted in any way to the inhibitor. Twenty-two per cent showed degenerative changes which followed 24 hours of normal growth; 20 per cent were completely inhibited. Although the percentage of partial inhibition was not high (16 per cent) at this level, it was very striking when it did occur. The difference between a control and a partially inhibited culture is shown in Figures 3 and 4.

The percentage of explants in 0.015 mgm/cc concentration which degenerated as a result of hexenolactone treatment was almost the same as in 0.025 mgm/cc concentration. A healthy control culture and a degenerating experimental culture are shown in Figures 5 and 6. Hexenolactone at 0.015 mgm/cc concentration did not inhibit any explants. At this level it was more effective in partially decreasing the amount of the outgrowth than at 0.025 mgm/cc. At the 0.011 mgm/cc concentration, partially inhibited fibroblasts were observed in 63 per cent of the cases. This proved to be the most effective concentration for partial inhibition.

2. *Effect on Explants of Epithelial Cells and Fibroblasts*

Hexenolactone completely inhibited all outgrowth from cultures of chick metanephros, set up in 0.5 mgm/cc and in 0.1 mgm/cc concentrations (Table 2). The excellent growth of the controls in this series indicates that the lack of growth was caused by the inhibitory action of the hexenolactone and not by a failure of the tissue to grow in vitro. In the lower concentrations (0.05 mgm/cc and 0.035

TABLE 2
EFFECT OF HEXENOLACTONE OF STOMACH, INTESTINE, AND METANEPHROS

Concentration hexeno-lactone mgm/cc	Number of explants	Percentage explants totally inhibited	Percent-age explants degenerated	Percentage explants differentially inhibited	Percent-age explants unaffected
0.5	23	100			
0.1	24	100			
0.05	91	70	8	2	20
0.035	112	43	8	1	48
0.025	137	20	14	13	53
0.02	16	25		6	69
0.015	76	18	28	13	41
0.011	14			7	93
Controls	259	12	2	6	80

mgm/cc) the results indicate that most of the explants were either totally inhibited or entirely unaffected. Relatively few showed degenerative changes or differential inhibitory effects. Explants of stomach and intestine, which, like metanephros, are a combination of epithelial cells and fibroblasts, seemed more susceptible to the inhibitory action of hexenolactone at 0.05 mgm/cc than did heart and leg muscle cultures. However, the large amount of the inhibition (70 per cent) in intestine and stomach explants was total. No differential effect was evident. Similarly, at 0.035 mgm/cc, stomach and intestine were more inhibited than heart and leg muscle fibroblasts at this same concentration. Again, the inhibition was not differential. Forty-three per cent of the cultures never produced any outgrowth of epithelial cells or fibroblasts, and 48 per cent of the cultures resembled controls in quantity and quality of outgrowth.

The degenerative changes that appeared in the fibroblasts in outgrowths of stomach and intestine explants were similar to those observed in heart and muscle cultures. However, in epithelial sheets, the degeneration was limited to fatty deposition. There was no evidence of cell disintegration. The degenerative effects (2 per cent) in control cultures likewise consisted only of an accumulation of fat in both fibroblasts and epithelial cells.

The growth of fibroblasts of a human mammary gland carcinoma, cultured in hexenolactone in 0.05 mgm/cc, 0.035 mgm/cc, 0.025 mgm/cc, and 0.015 mgm/cc concentrations, was strikingly inhibited (Table 3). During the three days duration of this experiment, no epithelial tongues or sheets appeared in the outgrowth of the controls or experimentals. In a scirrhous tumor of this type, the latent period of epithelial cells is often longer than that of fibroblasts. Therefore,

TABLE 3

EFFECT OF HEXENOLACTONE ON MAMMARY GLAND CARCINOMA

Concentration hexeno-lactone mgm/cc	Number of explants	Percentage explants totally inhibited	Percentage explants partially inhibited	Percentage explants unaffected
0.05	23	100		
0.035	20	80	20	
0.025	19	58	42	
0.015	15	40	60	
Controls	30	30		70

it cannot be concluded that the hexenolactone had a differential action upon the tumor components. However, it was evident that at the higher concentrations of hexenolactone, fibroblast growth was either diminished or completely inhibited. When experimental cultures did proliferate, the amount of growth was always less than the controls. (Figures 7 and 8.)

3. *Protective Effect of Serum*

Since the high percentage of the total number of all explants treated with hexenolactone appeared refractory to its differential inhibitory effect, it was thought that possibly the fetal cord serum in the medium exerted a protective effect, and thus decreased the potency of the synthetic lactone. In a series of experiments to test this, all heart explants set up in Gey's solution alone at 0.011 mgm/cc concentration were partially inhibited whereas, only 33.3 per cent of the heart explants cultured in serum and 0.011 mgm/cc hexenolactone were affected.

4. *Reversibility of the Effect*

One of Medawar's (1937) criteria of an inhibitor as compared with a toxic factor is the reversibility of the action of the inhibitor. Two series of experiments with the synthetic hexenolactone in concentrations from 0.05 mgm/cc to 0.015 mgm/cc indicated that the effects produced by these samples were likewise reversible within limits. In 24 hours, 80 per cent of the previously inhibited explants started to grow. In Figure 9, which is the same culture as Figure 2, but 24 hours after control media had been added, new cells had begun to emerge from the explant.

D. Discussion

The results of these experiments showed certain similarities to and differences among the results of other investigators who tested hexenolactone on tissue cultures. Accurate comparison is difficult because the published literature does not indicate whether or not all explants are affected at the inhibitory concentrations. Similar to the results of Medawar (1937), it was found here that above a certain concentration of hexenolactone (0.1 mgm/cc, in these experiments) outgrowths were totally inhibited. The optimum concentration for effec-

tive partial inhibition of heart fibroblasts was 0.011 mgm/cc. At this concentration Kuhn, Jerchel, Moewus, Möller, and Lettre (1943) report complete inhibition of heart fibroblasts.

Moreover, in this laboratory, hexenolactone failed to show a differential effect on cultures of stomach and intestine. None of the concentrations used completely inhibited fibroblasts without inhibiting epithelial cells at the same time. The age of the tissue seemed to have no effect.

The results of these experiments indicate that culturing tissue for 48 hours in concentrations from 0.015 mgm/cc to 0.05 mgm/cc hexenolactone produces a deleterious effect upon fibroblasts and epithelial cells which, during the first 24 hours, appeared normal. The degeneration was more pronounced in fibroblasts. This could be considered a toxic effect and in this respect the synthetic hexenolactone differs from the naturally occurring form (Medawar, 1937) which gave no indication of cytological damage to treated cells.

It is possible that the variations in the inhibitory effects recorded here are a result of the presence of fetal cord serum in the culture media. This is substantiated by the tissue culture experiments with and without serum and by Hauschka's (unpublished data) experiments on *Dugesia tigrina*. When cultures of *Dugesia* were exposed to hexenolactone and saline, the lactone showed 100 per cent activity as compared with 73.5 per cent activity when exposed to hexenolactone and fetal cord serum. The work of Hauschka and the tissue culture experiments here recorded indicate that fetal cord serum has a protective action against the hexenolactone. Further experiments have shown that α-alanine, β-alanine (Hauschka, 1944) and cysteine (Hauschka, Toennies, and Swain, 1945) decrease the action of hexenolactone. It seems possible that the different lots of fetal cord serum used during the period of the tissue culture experiments contained varying amounts of anti-hexenolactone substances such as cysteine and the alanines. This might account for the lack of uniformity in the results. Briggs (unpublished data) attributed his negative results on the effect of hexenolactone on tumor bearing mice to the possible chemical transformation in vivo of active hexenolactone into an inactive derivative.

Although under the conditions of these experiments, hexenolactone appears to have no technical value in culturing pure epithelial outgrowths, it has an inhibitory effect on fibroblasts in vitro.

E. Summary

A total of 1369 explants of chick embryo heart and leg muscle, intestine, stomach, and metanephros and of a human mammary gland carcinoma were grown in roller tube and hanging drop cultures in concentrations of hexenolactone ranging from 0.011 mgm/cc to 0.5 mgm/cc. Hexenolactone caused partial inhibition of the growth of heart and muscle fibroblasts, degenerative cytological changes, and, in high concentrations, total inhibition of all outgrowth.

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BOOK REVIEW

(F. E. Chidester. Nutrition and Glands in Relation to Cancer. New York: Lee Foundation for Nutritional Research, 1944. Pp. 247.)

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This amazing book is a highly personalized attempt to prove that the element iodine is the be-all and end-all of cancer. Prospective readers should know in advance that every last bit of fact, fancy, hearsay, and opinion provided by sacred and profane literature has been used by the author to bolster his thesis. Sans peur et sans reproche. And that the references thereto are largely as follows: "Colloidal iodine has been used with success by a distinguished clinician since 1925, and he reports cures up to 12 years standing in certain types of cancers." "Eppinge—in 1931, and Cohen—in 1935 have used thyroid extract with marked success in cutaneous ulcers." The way in which these and thousands of other items are woven into a pattern of the author's imagining is intriguing and on occasions breath-taking. For those interested in such compilations this book will be most satisfying.